Environmental Temperature and Relative Humidity, two Key Factors in Maize Technology Affecting Ochratoxin a Production and Growth of Ochratoxigenic Species

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***Abstract*—Ochratoxin A (OTA) is a secondary fungal metabolite produced naturally by filamentous fungi of the *Aspergillus* and Penicillium genera. The aims of this study were to identify the most relevant OTA producing species in maize in Spain by PCR and to study the effect of environmental conditions (a_w, temperature) on the development of these fungi and on OTA production in maize. Eighteen and 20 corn samples harvested in 2014 and 2015, respectively, from conventional farms and 31 and 34 corn samples harvested in 2014 and 2015, respectively, from organic farms located in various Spanish areas were tested for presence of ochratoxin-producing fungi. The most important OTA-producing *Aspergillus* spp. were isolated and identified by species-specific PCR protocols. The growth rate of the main ochratoxigenic species (*A. steynii* and *A. ochraceus*) under different environmental conditions was registered and OTA level in cultures was determined by solvent extraction, immunoaffinity column clean-up and LC-fluorescence detection. The results revealed that *A. steynii* and *A. ochraceus* are good indicators of OTA risk in maize. Temperature, a_w, and isolate influenced OTA production. The highest OTA levels were found in maize cultures of *A. steynii* at 0.98 a_w and 30 ℃.**

***Index Terms*—Ochratoxin A, ochratoxigenic *Aspergillus*, maize, environmental conditions, food safety, risk assessment**

I. Introduction

In spite of many years of research and the introduction of good agricultural practices in the food production, and good manufacturing practices in the storage and distribution chain [1], fungi and mycotoxins continue to be a problem in food and feed [2].

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It is estimated that 25% of the world's food production, including many basic foods, is affected by mycotoxin-producing fungi. Reports on mycotoxin occurrence on different cereals and by-products show that maize is the first most contaminated crop worldwide [3], [4].

Ochratoxin A (OTA) is produced by *Aspergillus* spp. sections *Circumdati* and *Nigri* in warm and tropical countries and in temperate climates it is produced by *Penicillium verrucosum* [4], [5]. The most important ochratoxigenic *Aspergillus* spp. are *A. ochraceus*, *A. westerdijkiae* and *A. steynii*, which belong to *Aspergillus* section *Circumdati* [6]. The second OTA-producing *Aspergillus* group is classified under *Aspergillus* section *Nigri* [7]. *Aspergillus* of sections *Circumdati* and *Nigri* have been isolated from cereals [8]-[11]. The main sources of daily OTA intake are cereals and cereal products, followed by wine, grape juice and coffee [8]-[13].

OTA is one of the most important mycotoxins in food and feed. IARC has classified this mycotoxin as a probable human carcinogen, in group 2B [14]. Previous studies have shown that a number of diseases are associated with OTA exposure in both animals and humans, predominantly affecting the kidney. It is also associated with other toxic effects such as neurotoxicity, reproductive toxicity, myelotoxicity, immunotoxicity, and teratogenicity [15]-[17].

In Spain, Mateo *et al.* [8] observed a high occurrence of *Aspergillus* spp. of sections *Circumdati* and *Nigri* in barley crops, which represent up to 40% of the Spanish cereal production. The presence of these fungi in pre- and postharvest barley grains indicates their adaptation to warmer weather. Magan *et al.* [18] suggested that climate change toward hot temperatures and drought could increase the risk of pathogen migration.

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The health-beneficial properties of maize have led to an increase in the consumption of maize-based food products in recent years. Due to the dietary importance of maize and its by-products it is worth to know and to control the ochratoxigenic mycobiota of this cereal, its distribution in the different agro-climatic regions worldwide and the environmental conditions that affect mycotoxin production in the cereal. The presence of a particular ochratoxigenic mould does not always indicate that OTA occurs. The contrary situation is also true: since OTA is stable, it might be detected long after the producing fungi have died out or have been outgrown by other species [3], [19], [20].

The interest in the area of food safety is increasing and traditional methods for detection and identification of possible ochratoxigenic fungi in food is laborious and often difficult even for expert taxonomists. New methods based on the Polymerase Chain Reaction (PCR) are rapid, specific and highly sensitive, being able to detect target DNA molecules in complex mixtures even when the fungi are no longer viable. Several PCR assays have been developed to detect OTA-producing *Aspergillus* spp. using as target constitutive genes or toxin biosynthetic genes [21], [22]. However, the sensitivity of the assay can be considerably improved when multi-copy sequences, such as ribosomal DNA regions, are used. Both ITS1 and ITS2 (Internal Transcribed Spacers or the rDNA) regions are highly variable in *Aspergillus* genus allowing discrimination among closely related species [23].

The aims of this work were: i) To determine the most important Aspergillus spp. that are potential producers of OTA in contaminated Spanish maize samples by species specific PCR protocols based on multi-copy sequences (ITS); and ii) To test the impact and effect of environmental conditions associated to storage on both the development of these ochratoxigenic moulds and OTA production in maize.

II. Materials and Methods

A. Mycological Study of Positive Samples

During 2014 and 2015 a total of 103 samples of corn were collected from 16 Spanish farms: 38 samples of corn were collected from 7 farms using conventional cultivation methods, and 65 samples were collected from 9 organic farms. These maize samples were collected in farms that showed a strong desire to cooperate in the present study and facilitated regular assistance with sampling. On the other hand, maize grains from organic crops were more susceptible to fungal attack and exhibited more variability on particular agricultural practices. Thus, a larger number of samples from organic crops was examined to generate adequate and sufficient database.

No samples were mouldy in appearance. The farms were located in wetland Spanish areas (Galicia, Asturias, Santander, Basque Country and "Albufera" of Valencia), where maize grows without artificial irrigation, and in dry areas or irrigated areas (Aragón, Castilla, Extremadura and Andalucia). Maize samples were taken 1 to 3 weeks after harvest. Sampling was performed following the protocol described in Commission Regulation (EC) No 401/2006. Representative subsamples of 1000g each were collected from each lot of cereal and homogenized to obtain a final sample of 10kg. The samples were sent to the laboratory and final subsamples of 500g were prepared and immediately processed for mycological analysis.

B. Isolation and Identification of Ochratoxigenic Fungi and Determination of Ochratoxin Producing Potential of the Isolates

To reduce surface contamination 100 g of each maize sample were surface-sanitized with 0.5L of a 2% solution of sodium hypochlorite for 1 min. Afterwards, the grains were washed twice with 0.25L of sterilized pure water for 1min. Fifty seeds per sample were placed on 9 cm diameter Petri dishes (5 seeds/dish) filled with Dichloran Chloramphenicol Peptone Agar (DCPA) [16] modified using 15% less quantity of chloramphenicol and incubated at 25 ℃ for 7 days. Based on their phenotypic characteristics, colonies identified at the section level as *Aspergillus* spp. section *Circumdati* and *Aspergillus* spp. section *Nigri* were selected and single spore cultures were prepared in Czapek Agar for identification. After 7 days, identification was carried out by species-specific PCR protocols.

OTA-producing *Aspergillus* species, *A. westerdijkiae*, *A. ochraceus*, and *A. steynii* following the methodology reported by Gil-Serna *et al.* [6]. PCR assays specific for these OTA-producing species, on the basis of the multicopy ITS regions of the rDNA were used. All genomic DNAs used in this work were tested for suitability for PCR amplification using primers ITS1 and ITS4, which amplify the ITS1-5.8S-ITS2 region. The specific PCR amplification protocol to detect *A. westerdijkiae*, using specific primers set WESTF/WESTR was as follows: 5 min at 95 ℃, 22 cycles of 30s at 95 ℃ (denaturalization), 30 s at 63 ℃ (annealing) and 40 s at 72 ℃ (extension) and, finally, 5 min at 72 ℃. OCRAF/OCRAR primers were used to detect *A. ochraceus* according to the following PCR program: 5 min at 95 ℃, 24 cycles of 30 s at 95 ℃, 30 s at 62 ℃ and 40 s at 72 ℃ and, finally, 3 min at 72 ℃. For *A. steynii*, STEYF/STEYR primers were used. In this case, the amplification protocol was as follows: 5 min at 95 ℃, 21 cycles of 30 s at 95 ℃ (denaturalization) and 45 s at 72 ℃ (annealing and extension) and, finally, 5 min at 72 ℃. WESTF/WESTR and OCRAF/OCRAR primers amplified a single fragment of about 430 bp only when genomic DNA of *A. westerdijkiae* or *A. ochraceus* was present, respectively. STEYF/STEYR primers amplified a single fragment of 315 bp only when genomic DNA of *A. steynii* isolates was present.

A. carbonarius, *A. niger*, and *A. tubingensis* were identified following the methodology reported by Medina *et al*. [7] and González-Salgado *et al*. [24]. *A. carbonarius* can be microscopically distinguished by conidial size and ornamentation and by PCR protocols using the CAR1/CAR2 primer pairs [24]. However, all the taxa in the *A. niger* aggregate are morphologically indistinguishable. Identification of isolates of *A. niger*

aggregate (*A. niger* and *A. tubingensis*) was based on PCR amplification of 5.8S rRNA genes and the two intergenic spacers, ITS1 and ITS2, followed by the subsequent digestion of these PCR products with restriction endonuclease RsaI [7]. Digestion was carried out overnight at 37 ℃ with RsaI (Boehringer Mannheim). PCR products and restriction fragments were separated by electrophoresis in 1% and 2% agarose gels, respectively, with 0.5 × Tris-borate-EDTA buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 μg/ml) and the DNA bands were visualized. The PCR-amplified-5.8S rRNA gene of *A. niger* is digested into two fragments of 519 and 76 bp. The 76-bp fragment was too small to remain on the gel. The PCR-amplified-5.8S rRNA gene of *A. tubigensis* was not digested by RsaI. In this latter case a band of 600 bp is observed.

C. Effect of Environmental Conditions on Growth and OTA production by A. steynii and A. ochraceus in Maize

Three isolated of *A. steynii* (AsM02, AsM11, and AsM09) and three isolates of *A. ochraceus* (AoM07, AoM05, and AoB13) were selected to study the effect of environmental conditions on fungal growth and OTA production in maize grains. These strains are held in the Mycology and Mycotoxins Group Culture Collection (Valencia University, Spain).

D. Growth Evaluation

Maize kernels (15g), previously analyzed to ensure they had undetectable levels of OTA were placed in Erlenmeyer flasks and autoclaved for 20 min at 121 ℃. Then, water activity (a_w) was adjusted to 0.96 and 0.98 by addition of sterile pure water. Flasks containing maize grains were refrigerated at 4 ℃ for 48 h with periodic shaking to allow for water adsorption and equilibration. At the end of this period, a_w-values were checked. Fifteen g of the hydrated maize grains were placed in sterile 9-cm Petri dishes to form a layer. All treatments were centrally inoculated with 3-mm diameter agar plugs taken from the margin of 5-7-day-old growing colonies of *A. steynii* and *A. ochraceus*. Inoculated Petri dishes of the same a_w were enclosed in sealed plastic containers together with beakers of a glycerol-water solution matching the same a_w as the treatments to maintain a constant equilibrium relative humidity inside the boxes. The experiments were carried out at two temperatures, 20 ℃ and 30 ℃, for 14 days. The experiments were carried out in triplicate and repeated once. Assessment of growth was made every day during the incubation period by measurement of two diameters of the growing colonies at right angles to each other until the colony reached the edge of the plate. The radii of the colonies were plotted against time, and linear regression was applied. The growth rate (mm/day) was estimated by the slope of the line. After 14 incubation days, three replicates per treatment were dried at 50 ℃ for 24h, milled, and stored at -20 ℃ before OTA determination.

E. OTA Determination

To find OTA level in maize seed cultures, all the kernels distributed as a homogenous layer on each Petri dish were used regardless of the colony diameter reached after 14 incubation days. Grains were milled, homogenised and analyzed for OTA by an optimized and validated method involving accelerated solvent extraction, clean-up by immunoaffinity column, liquid chromatographic separation and fluorescence detection, as described by Mateo *et al*. [8].

A Dionex Accelerated Solvent Extraction (ASE 150) system (Dionex Co., Sunnyville, CA) was used for extraction of OTA. Ten grams of ground sample was weighed and mixed with 3 g of diatomaceous earth drying matrix (Dionex). The sample was packed into the 22-ml extraction cell, and the void volume on the top was filled with glass beads. A mixture of acetonitrile–water (60:40, v/v) was used for OTA extraction. Temperature was 100 ℃ and pressure was 10.3 MPa. Fifty mL of extract (2×25 mL) was collected and let stand overnight at 4 ℃ to allow for protein precipitation. After filtering through paper filter the clear extract was concentrated in a rotary evaporator to near dryness and re-dissolved with acetonitrile-water (60:40, v/v) to a final volumen of 10 mL. Then, 2.5 mL was transferred to a 50-mL measuring flask and diluted with Phosphate-Buffered Saline (PBS) solution (2.5 g sample/50 mL). Clean-up was carried out using OchraTest immunoaffinity columns (Vicam, Watertown, MA). The column was first conditioned with PBS (20 mL). Then, 20 mL of diluted sample extract (1.0 g sample) was placed on the column and made to flow at a rate of no more than 3 mL/min. The column was washed with 10 mL pure water (Milli-Q) and then dried by passing air through the column (approximately 3 mL) to remove any remaining water prior to quantitative elution. OTA was eluted with methanol (4 mL). The solvent was carefully evaporated under a slight stream of N_2 at 40 ℃. The residue (1.0 g sample) was dissolved in 0.5 mL methanol–2% aqueous acetic acid (1:2), filtered through 0.22 μm filter into a vial and used for liquid chromatographic analysis.

Chromatographic conditions were as follows: the LC system consisted of a Waters 600 pump, a Waters 717 automatic injector and a Waters 474 scanning fluorescence detector (Waters Co., Milford, MA, USA). Separation was performed on a Phenomenex Gemini C18 column (150 × 4.6 mm, 5 μm particle size) (Phenomenex Inc., Torrance, CA, USA). The LC system was controlled and signals were processed by Millennium 32® software version 3.01.05 (Waters). Column temperature was 35 ℃. Separation was carried out at a flow rate of 1 mL/min using water-acetonitrile-acetic acid (41:57:2, v/v/v) as a mobile phase. The mobile phase was filtered through a 0.45-μm filter and degassed before use. Retention time for OTA was 5.1 min. Excitation and emission wavelengths were 330 and 460 nm, respectively.

F. Statistical Analysis

The data were treated by multifactor ANOVA and Duncan's test of multiple comparisons using Statgraphics Centurion XV.2.11 software (Statpoint Inc., VA, USA). A 95% confidence level was used to assess influence of individual and interacting treatments. For calculation purposes, detectable OTA levels below the limits of

quantification (LOQ) were estimated as 50% of those limits and undetectable levels were assumed to be zero. Logistic regression of OTA presence (0 = below LOD, 1 = above LOD) against presence of target DNA of ochratoxigenic *Aspergillus* spp. was carried out by categorizing the DNA variable as 0 = not detected or 1 = detected.

III. Results and Discussion

The *Aspergillus* spp. isolated from maize kernels and their ability to produce OTA are summarized in Tables I and II. Kernels were also infected by other fungal species belonging to the genera *Fusarium*, *Alternaria*, *Cladosporium*, *Epicoccum*, *Mucor* and *Rhizopus*.

Identical fungal genera were isolated from corn grown in both farming systems and geographic regions.

The percentage of infected samples with fungi was 100% in all cases. The predominant fungus in maize samples was *Alternaria* spp., followed by *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp., which agrees with results reported by other authors [25]-[27].

The occurrence *of Aspergillus* in seeds was higher in dry than in wet regions. Environmental conditions in dry regions offer obvious advantages to more xerotolerant fungi *Aspergillus* spp. over other less competitive species. Desertification and fluctuations in wet-dry cycles, associated to climate change will have a strong impact on the life cycle of all microorganisms including toxigenic moulds and their patterns of mycotoxin production in crops. In terms of total maize kernels infected with *Aspergillus* spp. ANOVA showed that there were significant differences ($p < 0.05$) with regard to the geographic origin of the samples and production system (organic or conventional) (Table I).

Only three ochratoxigenic species were identified by PCR protocols in the analyzed maize samples: *A. niger*, *A. steynii* and *A. ochraceus.* OTA producing potential of the isolates from these species is shown in Table II. OTA was produced mainly by *A. steynii* isolates following by *A. ochraceus* and *A. niger* isolates. The percentage of OTA producing isolates from conventional and organic maize farms was for *A. steynii*, 87.5 and 100%; for *A. ochraceus* 33.3 and 36.4%, and for *A. niger* 30.0 and 33.3 %, respectively. Moreover, very low OTA concentrations were found in *A. niger* cultures and frequently such levels varied between the LOD and LOQ (0.05 and 0.17 ng/g, respectively). These results lead to the conclusion that *A. steynii* and *A.* ochraceus are the main species involved in the production of OTA in the studied maize samples.

TABLE I. *Aspergillus* spp. Occurrence in Conventional and Organic Maize Samples Harvested in 2014 and 2015 in Different Spanish Regions

Spanish region	Production system	No. of samples 2014+2015	Total No. of kernels	% Infected kernels mean (range)
Wet regions	Conventional	10+11	1050	5 (1-14)
	Organic	19+21	2000	10 (5-13)
Dry regions	Conventional	8+9	850	13 (1-22)
	Organic	12+13	1250	18 (5-28)

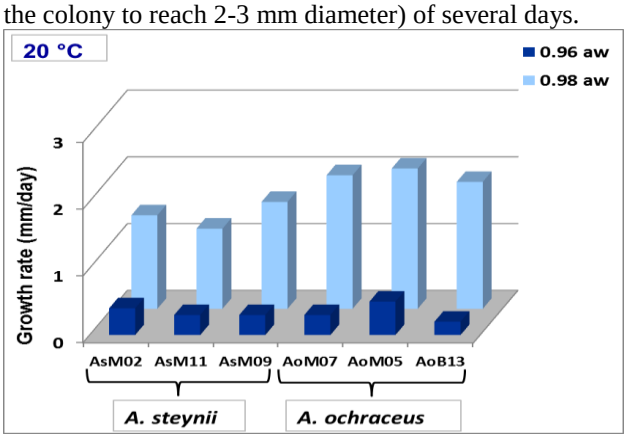
TABLE II. Ochratoxin A Producing Potential of Fungi Isolated from Conventional and Organic Maize Samples Harvested in Different Spanish Regions

Fungi	Toxin	No. of assayed isolates (% positive)	
		Conventional maize	Organic maize
A. niger	OTA	10 (30.0)	12 (33.3)
A. steynii	OTA	8 (87.5)	5 (100.0)
A. ochraceus	OTA	6 (33.3)	11 (36.4)

The effect of environmental conditions on the growth rate and OTA production of three selected isolates of *A. steynii* and *A. ochraceus* in maize can be observed in Figs. 1 and 2. ANOVA revealed that a_w and temperature and their interaction had a significant effect on growth responses ($p < 0.05$). In general, growth was faster at 30 ℃ than 20 ℃, regardless of a_w, and at 0.98 than 0.96 a_w, regardless of temperature. Under the same conditions, no significant differences were found among the isolates of both species regarding growth kinetics (Fig. 1).

Fig. 2 shows the effect of temperature and a_w on OTA concentration (μg/kg) in all maize cultures of the six isolates under all the assayed conditions. ANOVA showed that a_w, temperature and isolate and their two and three way interactions had a significant effect on OTA production ($p < 0.05$). Usually, OTA levels were much higher at 30 ℃ than 20 ℃, regardless of a_w, and at 0.98 than 0.96 a_w, regardless of temperature, and higher by *A. steynii* than *by A. ochraceus* regardless of a_w and temperature. The highest OTA levels were found in maize cultures of *A. steynii* at 0.98 a_w and at 30 ℃.

No previous report has examined the impact that environmental conditions have on growth of *A. steynii* and *A. ochraceus* isolated from Spanish maize and on OTA production in maize kernels. As far as we know, only a study on wheat has been published [28] using ochratoxigenic isolates obtained from other hosts (barley and grapes). In that paper [28], very high OTA levels were found in wheat cultures of *A. steynii* incubated at 25/35 ℃ cycle, which surpass 1 mg/kg. The assays were performed by inoculation of seeds on the dish center with 5 μL of a spore suspension of fungi of 10^6 spores/mL. When cereal grains are used as culture media, this technique generally produces a lag phase (time spent for the colony to reach 2-3 mm diameter) of several days.



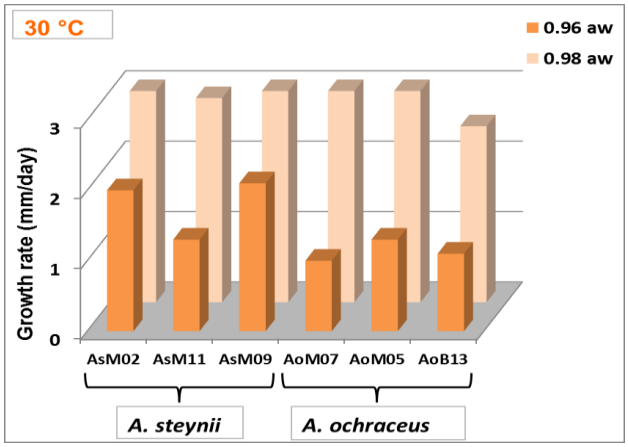


Figure 1. Effect of a_w, temperature and isolate on the growth rate of *A. steynii* and *A. ochraceus* isolates in maize cultures.

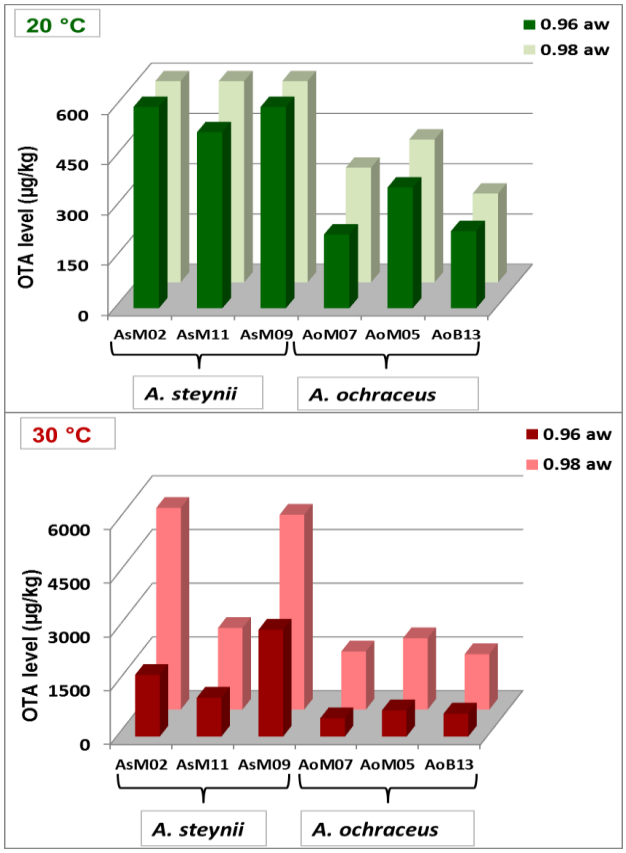


Figure 2. Effect of a_w, temperature and isolate on OTA production by *A. steynii* and *A. ochraceus* isolates in maize cultures.

However, those authors found average growth rates, for example, for *A. carbonarius*, higher than 7 mm/day during the first incubation week, which do not agree with results of the present study.

More studies are needed to clarify the effects of temperature and relative humidity on the growth of ochratoxigenic fungi and OTA production in cereals, as these commodities represent the main dietary source of OTA. Ecological studies on the effect of environmental conditions on growth and T-2 and HT-2 production by *Fusarium langsethiae* isolated from oats in England, Finland, Norway and Sweden have been carried out [29]-[31]. It was suggested that there is little difference between *F. langsethiae* strains from different European countries in terms of ability to grow under interacting extrinsic environmental factors but there are differences related to a_w and temperature. Comparative studies on growth profiles and mycotoxin production by *F. langsethiae* related to a_w and temperature in oat-based media and oats show that the growth rate and production of T-2 and HT-2 are significantly lower in the cereal than in culture media made with cereal extracts under all the assayed conditions [31], [32].

In summary, very few studies have examined the impact that interacting environmental conditions can have on growth of fungal pathogen and mycotoxin production in cereal grains. The present study has shown, for the first time, data on the influence of interacting ecological factors on growth and OTA production by *A. steynii* and *A. ochraceus* in maize. Both environmental factors, a_w and temperature, appear to be very important in determining accumulation levels of OTA in this cereal. Consequently, global warming and increasing rain will devise a scenario that will favour OTA production by ochratoxigenic species that contaminate maize, particularly, *A. steynii*, a high OTA producer.

Although the complete elimination of mycotoxins in maize is not achievable at this time, the results of the present study show that implementation of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) to minimize fungal growth and mycotoxin occurrence in the cereal is needed. A Code of practice for the prevention and reduction of mycotoxin contamination in cereals [33], was adopted in 2003, amended in 2014 and revised in 2016, in the frame of climate change. It includes recommendations to be adopted in different stages of the crop, such as planting and crop rotation, tillage and preparation for seeding, pre-harvest, harvest, drying and cleaning before storage, storage after drying and cleaning, transport from storage and processing and cleaning after storage.

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